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Determination of Constituents of Extract of *Celtis tournefortii* Lam. by LC-MS/MS, Investigation of Enzyme Inhibition, Antimicrobial and Anticancer Effects

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Abstract

Phytochemicals found in extracts obtained from plants are very important bioactive constituents. In this study, phytochemicals in extract content obtained from *Celtis tournefortii* Lam. tree (CT) leaves were determined by a LC-MS method. The constituents with the major concentrations was found rutin (2479.89 µg ml^{-1}), coumarin (1241.68 µg ml^{-1}), biochanin A (1026.42 µg ml^{-1}), shikimic acid (477.32 µg ml^{-1}), chlorogenic acid (300.76 µg ml^{-1}). The suppressive effects of CT extract on the growth of pathogenic strains were studied by microdilution method. It was observed that it caused suppression on the strains in the concentration range of 2.00-8.00 µg ml^{-1}. The inhibition effects of the extract on acetyl cholinesterase and glutathione-S transferase enzyme activities were investigated, and 50% inhibitory values of enzyme activity were found to be 13.58 and 13.86, respectively. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay method, the cell viability suppressive effects of CT leaf extract were studied in healthy skin fibroblast cells as well as ovarian, colon and brain cancer cells. It was observed that they created a 42%, 4.27%, and 14.29% suppression in cells, respectively.

Keywords: Anticancer, antimicrobial, *Celtis tuernofortii*, chemical composition, enzyme inhibition

Celtis tournefortii Lam Yaprak Özütünün Bileşenlerinin LC-MS/MS Aracılığıyla Belirlenmesi, Enzim İnhibisyonunun, Antimikrobiyal ve Antikanser Etkilerinin Araştırılması

Öz

Bitkilerden elde edilen özütlerde bulunan fitokimyasallar çok önemli biyoaktif bileşenlerdir. Bu çalışmada, *Celtis tournefortii* Lam. ağacı (CT) yapraklarından elde edilen özüt içeriğindeki fitokimyasallar LC-MS yöntemi ile belirlendi. Rutin (2479.89 µg ml-1), kumarin (1241.68 µg ml-1), biyokanin A (1026.42 µg ml-1), şikimik asit (477.32 µg ml-1) ve klorojenik asit (300.76 µg ml-1) bileşenlerinin yüksek konsantrasyonlara sahip olduğu belirlendi. CT ekstraktının patojen suşların büyümesi üzerinde baskılayıcı etkileri mikrodilüsyon yöntemi ile çalışıldı. 2.00-8.00 µg ml-1 konsantrasyon aralığında suşlar üzerinde baskılamaya neden olduğu gözlendi. Özütün asetil kolinesteraz ve glutatyon-S transferaz enzim aktiviteleri üzerindeki inhibisyon etkileri incelendi ve enzim aktivitesinin %50 inhibitör değerleri sırasıyla 13.58 ve 13.86 olarak bulundu. 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazol bromür tekniği uygulanarak CT yaprak özütünün hücre canlılığını baskılayıcı etkileri yumurtalık, kolon ve beyin kanseri hücrelerinin yanı sıra sağlıklı cilt fibroblast hücrelerinde çalışıldı. Kanser hücrelerinde sırasıyla %42, %4.27 ve %14.29 oranında baskılama oluşturduğu gözlendi.

Anahtar kelimeler: Antikanser, Antimikrobiyal, Celtis tournefortii.

INTRODUCTION

Plants have two types of metabolism, primary and secondary. While primary metabolism is used for vital activities, secondary metabolism and the secondary metabolites produced as a result of this

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metabolism are also important in the processes used in various stress situations (Płonka et al., 2020). Polyphenols, one of the secondary metabolites produced by plants, are products with very high benefits by playing a role in many biological activities in various living things, especially humans (Engström et al., 2015).

Due to their widespread presence in plants, bioactive substances like polyphenols have a significant role in human diets. (Wang et al., 2015). Plant-derived phenolic constituents that make up the color of many fruits and vegetables are very useful constituents. Potential health benefits include protecting low-density lipoprotein (LDL) from oxidation, preventing various age-related diseases, and antioxidant activity (Tiong et al., 2016). In addition, these phytochemicals have activities such as reducing collagen degradation and anti-aging, protecting from the harmful effects of UV (Nascimento et al., 2021). Various medical applications such as antimicrobial and anticancer have been made by using extracts obtained from different parts of plants (Moldovan et al., 2014; Ishaque et al., 2021; Mohan Reddy et al., 2021; Sinan et al., 2021)

Dementia, often recognized as Alzheimer's disease (AD), is a severe neuron disease that harms brain cells and results in irreversible memory loss. Since there is no treatment for this illness, it claims many lives every year; however, early discovery can lessen the spread of the illness. People over 65 are most likely to develop Alzheimer's disease. It is well recognized that amyloid-(A) peptide buildup in the brain plays a significant role in the pathophysiology of the illness. (Karran and De Strooper, 2022). Efforts to develop drugs that can slow or delay the progression of AD have long been the focus of studies in recent years. AD markedly differs in Acetylcholine (ACh) levels. A decrease is observed in the synthesis of ACh, which is used as a neuromediator (Jaramillo et al., 2022). The main reason for this is the decrease in the amount and function of the synthesized acetylcholine transferase enzyme, decrease in choline reuptake, damage in cholinergic neurons and axons. Retention of acetylcholine for neurotransmission in the synaptic cleft both reduces and prevents Alzheimer's symptoms (Aras et al., 2021).

Glutathione-S transferase (GST) is an enzyme that has important contributions to several mechanisms in the detoxification of xenobiotics in metabolic processes. In addition, the enzyme plays a role in the biosynthesis of molecules such as testosterone and progesterone and in the degradation of tyrosine (Hayes et al., 2005). Byproducts of endogenous ROS activity and molecules such as exogenous polycyclic aromatic hydrocarbons are electrophilic substrates of GST. This enzyme conjugates a wide variety of electrophilic xenobiotics with reduced glutathione, blocking their activation and removing them from the body (Strange et al., 2001). There are two active sites in the enzyme, the G site and the hydrophobic H site. The hydrophobic H section has a position covering two separate functional positions that can engage several electrophilic substrates, while a G region binds the physiological substrate. (Erat et al., 2008; Çomaklı et al., 2011).

C. tournefortii Lam. (CT) is a tree species of about five meters tall, which grows in temperate regions, which is called "Eastern fenugreek, Dardagan or Dagdagan", which is in the Cannabaceae family (Gecibesler, 2019; Yıldırım et al., 2017). There have also been reports of various actions in Celtis species, including antioxidant, anticancer, antibacterial, and anti-inflammatory effects, acetylcholinesterase (AChE) inhibitors, and some other properties of its chemical constituents. In studies conducted to evaluate the polyphenol contents for Celtis species, it has been shown that chemical ingredients such as alkaloids, coumarins, flavonoids, tannins, terpenoids, coumaroyl tyramines, amide compounds, steroids are present (Keser et al., 2017; Qi et al., 2021; Wang et al., 2022).

In this research, it was aimed to determine enzyme inhibition activities, antimicrobial and anticancer effects, the profile of chemical constituents of the extract obtained from CT leaves by the LC-MS/MS

MATERIAL VE METHOD

Plant Collection and Leaves Extraction

In the Kızıltepe region of Mardin, plant leaves were gathered at the end of the summer season from the location area shown in figure 1. It was subjected to washing (tap water and distilled water) several times. After washing, plant leaves taken to room conditions were left to dry on blotting paper. The dried plant leaves were ground into powder. To obtain the extract of CT leaves in methanol (MECT), 20 grams of dried leaves and 200 mL of methanol were

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mixed at room conditions and left for four days at 25 °C and filtered. The filtrate was taken from the methanol by means of a Heidolph 94200 rotary evaporator. The resulting content was stored at -18 °C for use in applications.

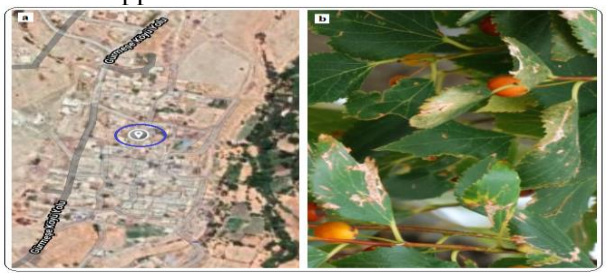

Figure 1. Belonging to the CT plant; Images of the location (a), and morphological appearance of the area where it grows (b)

AChE and GST Enzyme Inhibition

The inhibition activity of the extract prepared using CT leaves on the AChE enzyme in the concentration range of 5.00-25.00 µM was examined by UV-visible spectrophotometry (UV-Vis) method (Ellman et al., 1961). The commercial form of AChE (EC 3.1.1.7, Sigma) purified from Electrophorus electricus was used as the enzyme source in practice. Acetylcholine iodide (AChI) was used as a substrate in the cholinergic reaction. A Tris/HCl buffer was adjusted to 1.0 M pH 8.0 and extract solutions prepared in the range of 10-30 µg mL^{-1} were transferred to 0.5 mM 50 µL AChE enzyme solution ($5.32x10^{-3}$). The mixture solution was kept at 20 °C for 10 minutes. Then, 5,50-dithio-bis 2-nitro-benzoic acid (DTNB) was added to the mixture and the reaction was started for enzyme activity. AChE activity was measured at a wavelength of 412 nm, enzyme activity evaluations were made using spectrophotometry data, and the IC_{50} value was calculated (Ahmed et al., 2006; Behera and Bhatnagar, 2018).

An aromatic electrophile and a glutathione molecule couple through the action of the enzyme GST. The electrophile in the aromatic group, 1-chloro-2,4-dinitrobenzene, is the most often employed substrate for this reaction (CDNB). The dinitrobenzene S-glutathione (DNB-SG) formed as a result of the reaction gives maximum absorbance at UV-vis 340 nm wavelength. The IC50 value was determined by evaluating the absorbances obtained at UV-vis maximum wavelength by using CDNB in phosphate buffer at room temperature to inhibit the activation of the GST enzyme by the leaf extract of CT (Lineweaver and Burk, 1934; Liu et al., 2014; Gülçin et al., 2016).

Utilizing LC-MS/MS to Determine Phytochemical Constituents

Material and reagents

Commercially available items included analytical-grade phenolic standards, ammonium formate, formic acid, methanol, and acetonitrile from Sigma-Aldrich. Millipore's Milli Q pure water gadget was used to prepare the pure water that was used for chromatographic purposes. A captiva premium syringe filter with a nylon membrane, a polypropylene housing, a diameter of 25 mm, and a pore size of 0.45 m was used to filter all solutions prior to analysis.

Mass spectrometry and chromatography conditions

Qualitative evaluation of constituents was performed using High-performance Liquid Chromatography (HPLC) model 1260 Infinity II LC system with integrated tandem mass spectrometry. A degasser, a column furnace and dual pumps were integrated into the reverse phase HPLC device. In order to segregate molecules and overcome suppression effects, chromatographic settings were improved. At 25 °C, chromatographic separation for optimization was carried out using an analytical column with the reversed phase Poroshell 120 EC-C18 type (100 mm x 3.0 mm, 2.7 m). The elution gradient, solvent flow rate, and injection volume were adjusted to 0.4 mL min-1 5 µL, 5 mM ammonium formate in water (selective: A) and 0.1% formic acid in acetonitrile (selective B). The gradient elution profile utilized was as follows:

10% B (0−1 min), 40% B (1−3 min), 70% B (3−5 min), 40% B (5−6 min), and 10 % B (6−8min).

Using an electrospray ionization (ESI) source and scanning of ion changes from the primordial phytochemical to the moiety identified by the MRM technique, phytochemical compounds were specifically detected by LC-ESI-MS/MS.

Antimicrobial Suppressing Effects of CT Leaf Extract on Hospital Pathogens

Inhibitory effects of CT extract on the growth of hospital pathogens, *Staphylococcus aureus* ATCC 29213 (S. aureus), *Bacillus subtilis* ATCC 11774 (B.

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subtilis), *Escherichia coli* (E. coli) ATCC25922, *Pseudomonas aeruginosa* ATCC27833 (*P. aeruginosa*), *Candida albicans* (*C. albicans*) were analyzed using the Micro dilution method (Baran et al., 2021; Baran, 2018; Baran et al., 2021). Minimum Inhibition Concentration (MIC) values affecting these microorganisms were determined by this method. All microorganisms were obtained from Artuklu University Microbiology Research Laboratory, Mardin, Turkey. Microdilution was performed on the microplate wells by starting from the first well with the medium and the CT leaf extract prepared at different concentrations. Some wells were reserved for other control steps of growth. In addition, the suppressive effects of antibiotics on the growth of pathogenic strains were examined with the same method, for comparison purposes. The Antibiotics were used for Vancomycin gram positive S. aureus and B. subtilis strains, Colistin for gram negative P. aeruginosa and *E. coli* strains, as well as Flocanozole antibiotics for C. albicans fungus. After applying the microdilution method, the microplates were incubated at 37 °C for 24 hours. At the end of the period, reproduction control was performed and MIC was defined.

Anticancer Effects of CT Leaf Extract on Cancer Cell Lines Suppressing Viability

The cytotoxic effects of the constituents in CT leaves were examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT) method (Aktepe et al., 2021; Atalar et al., 2021; Baran et al., 2021; Baran et al., 2021) in Dicle University Scientific Research Center, Cell Culture Laboratory, Diyarbakır, Turkey. In the experimental study, Colorectal Adenocarcinoma (Caco-2), Glioblastoma (U118), and Human Ovarian sarcoma (Skov-3) lines were used as cancer cell lines. In addition, cytotoxic effects on the Dermal Fibroblast (HDF) healthy cell line were also evaluated. The absorbance spectrum of the cells was examined using Multi Scan Go Thermo with the cells adjusted to a wavelength of 540 nm. By using these absorbances, the concentrations and IC_{50} values of CT leaf extract cells that suppressed percent viability were calculated using the equations 1 given below (Awad et al., 2019; Baran et al., 2021).

%viability=U/C*100 (1)

In equality; U, The absorbance values of the cells after the interaction of the plant extract, and C, is the absorbance values of the control cells in the absence of the plant extract.

RESULTS AND DISCUSSION

Phytochemical Profile of CT Leaf Extract

When the LC-MS/MS data are examined, the bioactive compounds found in the CT extract content with the highest concentrations are rutin (2479.89 µg mL^{-1}), coumarin (1241.68 µg ml mL^{-1}), biochanin A (1026.42 µg mL^{-1}), shikimic acid (477.32 µg mL^{-1}), chlorogenic acid (300.76 µg mL^{-1}), vanillic acid (200.77 µg ml mL^{-1}), quercetin-3-glucoside (198.68 µg mL^{-1}), 4-hydroxy benzoic acid (260.63 µg mL^{-1}), salicylic acid (160.18 µg ml^{-1}) was found to belong to molecules such as. With these Constituents in high concentration, the LC-MS/MS profile showed that the CT plant extract was also rich in other bioactive compounds (Figures 2 and 3).

It is a medically important bioactive compound with the highest concentration of rutin antitumoral, antidiabetic, antimicrobial, anti-inflammatory effects (Chen et al., 2016). Biochanin A is a bioactive compound from the isoflavone family, which has very positive effects on health such as anticancer activity, cardiovascular defense, antioxidant properties, anti-inflammatory properties (Sundaresan et al., 2018). Cinnamic acid conjugates are commonly found in dicotyledonous plants. Among them, the most studied and known are chlorogenic acids. p-coumaric, ferulic, and caffeic acids are a few examples of the trans-cinnamic acids that form ester linkages with chlorogenic acids (Clifford et al., 2007). Chlorogenic acids have an active role in the regulation and reduction of body weight and on antioxidant mechanisms. However, they are also involved in the suppression of obesity with their effects on lipid metabolism (Chen et al., 2016). Quercimethrin, Quercetin-3-glucoside 110.50 and 198.68 µg ml^{-1} were found in the CT leaf extract. Quercetin derivatives, which are among the flavonoids, have a very high therapeutic potential in various diseases such as influenza (Wach et al., 2007; Mighri et al., 2019). In a study using Plicosepalus acacia, the phytochemical profile of the extract was examined by LC-MS/MS and the positive effects of Quercetin due to diabetes ischemia was shown (Abdel-hamed et al., 2021). The role of plant-derived Caffeic acids in biological activity is very important. Among these activities, there are effects such as antioxidant, antimutagenic, antitumor, antiobosite

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(Wang et al., 2015). The amount of transferullic acid of CT extracts was determined as 109.36 µg ml^{-1}.

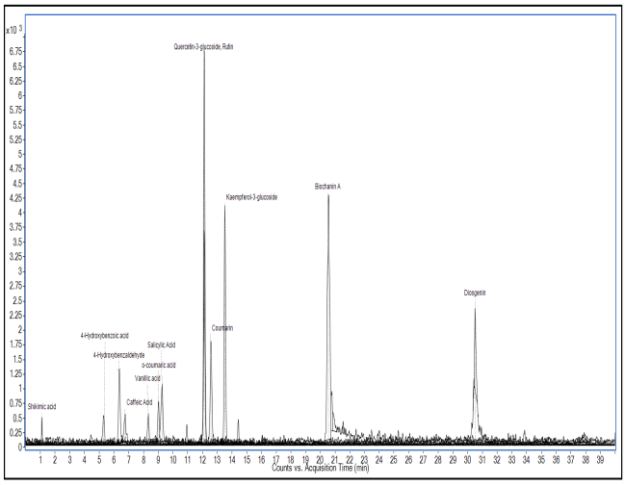

Figure 2. LC-MS/MS chromatogram of *CT* leaf extract

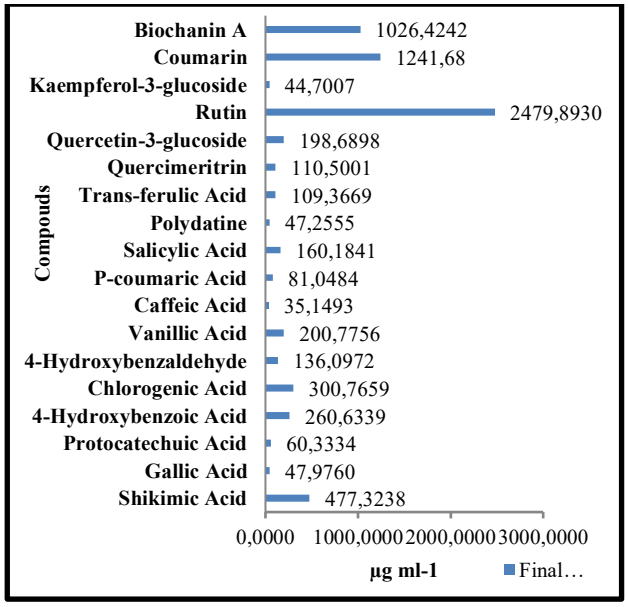

Figure 3. Bioactive compounds and their amounts found in LC-MS/MS profile of *CT* leaf extract

In a study with plant extracts of *Hibiscus roseus*, the profile of this compound was examined by LC-MS (Wang et al., 2015). *Ephedra alata* extract was found to be 1406.31 ± 35.74 µg mL^{-1} in LC-MS results (Mighri et al., 2019). It was determined that Protocatechuic acid was in the amount of 60.33 µg mL^{-1}in the compounds of the CT extract. In addition to its suppressive effect on platelet aggregation, protocatechic acid also has medicinal effects such as inhibiting apoptosis of human umbilical vein endothelial cells (Li et al., 2017).

Enzyme Inhibitory Activities of CT Leaf Extract

In enzyme activity studies performed in the range of 5.00-25.00 µM using the extract obtained from CT leaves, it was determined that the concentration range of 20-25 µM suppressed the enzyme activity by 75%-85% (Figure 4a and Table 1). By means of the enzyme activity data obtained in the range of 0.05-25.00 µM, the IC_{50} values on the AChE enzyme were calculated as 13.58. These results showed that the bioactive compounds found in high amounts in the plant extract greatly suppressed the AChE enzyme. In Romania, it was reported that the inhibition effect of the phytochemical content on the activity of the AChe enzyme in hydrosol extracts belonging to three species of Lamiaceae family plants was inhibited by 10.65% at 2.5 mg mL^{-1}, and 42.48% at mL and 5 mg mL^{-1} (Gaspar-pintiliescu et al., 2022).

The inhibition effect of CT extract on GST antioxidant enzyme activity was evaluated by UV-vis measurements in the range of 5.00-25.00 µM. Enzyme activity of 5-10 µM concentration range was detected at a rate of 87-57%. Using the measurements made, the IC_{50} values of the Constituents in the CT extract on the GST enzyme were determined as 13.86 (Figure 4 and Table 1). In a study conducted with methanol extracts of three different plant species, it was reported that *Terminalia bellerica* methanol extract had 73.96% and 82.29% GST enzyme activity at 5 and 10 mg ml^{-1} (Behera and Bhatnagar, 2018). In another study, it was shown that the IC_{50} values of phloridzin, baicalin, baicalein, and phloretin flavonoids obtained from natural plant sources on GST were 57.50, 28.75, 769.10, and 99.02, respectively.

Antimicrobial Effects of CT Extract

The highest concentration at which the CT extract was effective, 8.00 µg mL^{-1}, occurred on *E. coli*. At the lowest concentration, 2.00 µg mL^{-1}, the best effect occurred on the growth of P. aeruginosa bacteria. (Table 1, Figure 5). It is thought that the bioactive constituents with high concentration in the CT extract play a role in the antimicrobial effect (Chen et al., 2016; Santas et al., 2010).

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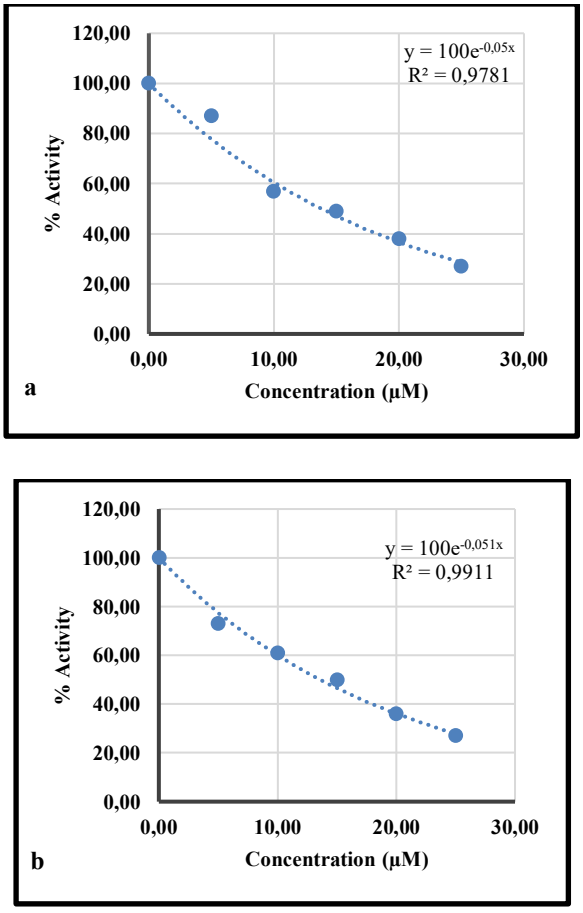

Figure 4. *CT* leaf extract; Inhibitory effect on AChE (a), and GST (b) enzyme activities

Table 1. *IC_{50} values of CT leaves extract due to inhibition of AChE and GST enzyme activities*

Compounds	AChE		GST	
	IC_{50}	R^2	IC_{50}	R^2
***CT* leaves Exracts**	13.58	0.9821	13.86	0.991

In the antimicrobial activity study conducted with *Gardenia latifolia* methanolic fruit extract, the MIC values effective on pathogenic strains *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* were 31.25 µg μL^{-1}, 31.25 µg μL^{-1} ,62.5 µg μL^{-1}, and 15.62 µg μL^{-1}, respectively. (Mohan Reddy et al., 2021). In another study, it was reported that *Pseudocedrela kotschyi* extract was effective on *S. aureus*, *E. coli*, and *P. aeruginosa* with MIC values of 0.3-0.7 mg ml^{-1} (Sinan et al., 2021). In addition to these, it has been stated that Juglans regia extract has an antifungal effect on *Candida* species in the concentration range of 137.50-275.00 µg mL^{-1} (D'angeli et al., 2021).

In studies conducted to examine the phytochemical compounds of plant extracts, it is thought that some molecules may play a role in the antimicrobial effect (antibacterial, antifungal, etc.). Because extracts are constituents rich in proanthocyanidins, which provide antimicrobial effect, and other molecules that will provide this effect (Rauf et al., 2019). The bioactive constituents in the plant extracts show suppressive activity by increasing the level of ROS in microorganisms, inhibiting biofilm, stimulating the apoptotic mechanism, interfering with the synthesis of important molecules such as chitin, glucan, glucosamine, ergosterol, proteins. In addition, the bioactive constituents in the extract also activate antimicrobial action mechanisms such as deterioration in the structure and functions of cell membranes, inhibition of enzymes such as DNA gyrase and protein kinase, dehydratase, and type III secretion inactivation (Rempe et al., 2017; Silva et al., 2021).

Table 2. *MIC values of CT leaves extract and antibiotics used to suppress the growth of microorganisms have antimicrobial effects*

TESTED ORGANISM	*CT* Leaves Extract µg mL^{-1}	Antibiotic µg mL^{-1}
S. aureus	6.00	2.00
B.subtilis	4.00	0.50
E. coli	8.00	4.00
P. aeruginosa	2.00	4.00
C. albicans	4.00	1.00

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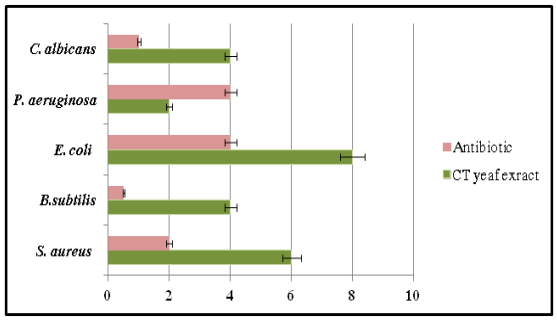

Figure 5. MIC values of CT leaf extract and antibiotics on the growth of microorganisms

Effects of CT Leaves Extract on Cancer and Healthy Cells

The effects of bioactive constituents in CT leaf extract on healthy cells and cancer cells were examined using the MTT method. It was determined that phytochemicals in high concentrations in the extract had a proliferative effect in healthy cells. However, the same dose of 250 g mL^{-1} extract had a proliferative effect on CaCo-2 cells while suppressing the viability of U87 and Skov-3 cancer cells by 18.41% and 37.09%, respectively. In the application where the extract concentration was 500 µg mL^{-1}, the viability of Skov-3, CaCo-2, and U87 cells was suppressed by 42.11%, 4.27%, and 14.26%, respectively. The IC_{50} values of HDF, Skov-3, U87, and CaCo-2 cells of the constituents contained in the leaf were determined as 196.23, 246.91, 213.76, and 349.65. According to a study, phenolic chemicals in *Rhus trilobata* extract to increase the level of ROS on skov-3 cells, which reduces cell viability. (Muthukumaran et al., 2013). It was stated that after incubation of *Juglans regia* (L.) extract on CaCo-2 cells for 48 and 72 hours, viability was suppressed by 40-50% (D'angeli et al., 2021). In another study, it was reported that a mixture of plant extracts of five different species suppressed viability by 50% at a concentration of 100 µg mL^{-1} on U87 cells (Omoruyi et al., 2021).

Bioactive compounds prevent proliferation, invasion, adhesion, tube formation in cancer cells. In addition to these, it has antiproliferative effects such as suppressing the metabolism of neoplastic transformation and chemical carcinogenesis by negatively affecting the DNA molecule (Aissani et al., 2021; Lu et al., 2010; Muthukumaran et al., 2013; Stefanowicz-Hajduk et al., 2021).

CONCLUSION

Plant extracts are rich sources of bioactive content such as natural polyphenols. These compounds have many beneficial effects. The components with the highest concentration found in the polyphenol content in the LC-MS/MS profile of the extract obtained from CT leaves, rutin, coumarin, biochanin A, shikimic acid, chlorogenic acid, vanillic acid, quercetin-3-glucoside, 4-hydroxybenzoic acid, salicylic acid, 4-hydroxy benzaldehyde, and quarsimethrin were determined. By using the microdilution method, it was found that the MIC values of the extract's constituent parts on the development of pathogen strains ranged from 2.00 to 8.00 g ml^{-1}.The IC_{50} values of the polyphenol content in the extract were determined to be 13.58 and 13.86 on AChE and GST enzyme activities. The suppressive effects of Skov-3, CaCo-2, and U87 cancer cells on the proliferation of healthy skin fibroblast cells were investigated by the MTT method. It was observed that the bioactive compounds of CT leaf extract were suppressive in cancer cells while promoting proliferation in healthy cells.

Bioactive compounds in plant extracts can be purified and their use in medicine can be studied in detail. Considering the positive effects of each component in pharmacology, it will contribute greatly to future studies.

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CONFLICT OF INTEREST

The Authors report no conflict of interest relevant to this article.

RESEARCH AND PUBLICATION ETHICS STATEMENT

The authors declare that this study complies with research and publication ethics.

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